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Purification of *Rhodobacter capsulatus* RNA polymerase and its use for in vitro transcription

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RNA polymerase from the photosynthetic bacterium *Rhodobacter capsulatus* has been purified to near homogeneity. The fractions that contained RNA polymerase activity were compared with *Escherichia coli* RNA polymerase in SDS-PAGE. The predominant bands were putative β and β' subunits of about 150–160 kDa, a candidate σ subunit of about 70 kDa, and a potential α subunit of approx. 45 kDa. This preparation was active in a run-off transcription assay with a linearized pUC13 plasmid as template, and specific transcripts were detected.

RNA polymerase; Transcription; *(Rhodobacter capsulatus)*

1. INTRODUCTION

Rhodobacter capsulatus (formerly known as *Rhodopseudomonas capsulata* [1]) is a Gram-negative facultatively photosynthetic bacterium, and is taxonomically grouped with the purple non-sulfur bacteria [1,2]. It is capable of growth with several distinct mechanisms of energy generation, including chemoheterotrophic (aerobic in the dark) and photoheterotrophic (anaerobic in the light) growth modes [2].

The regulation of expression of photosynthesis genes encoding light-harvesting I (B870) and reaction center complexes by oxygen has been shown to be at the level of RNA transcription [3]. However, the precise mechanism by which transcription is controlled is unknown.

Until now studies of *R. capsulatus* gene expression have been done in vivo [3,4]. The use of an in vitro transcription system would enable certain aspects of the expression of photosynthesis and other genes to be studied more rapidly and easily, and would complement the in vivo approaches. As the first step in development of an in vitro transcription system we have isolated RNA polymerase from *R. capsulatus*, and tested its activity with different templates.

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Abbreviation: SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Growth of bacterial cultures*

Cultures of *R. capsulatus* B10 were grown at 34°C in 20 l batches of RCV medium [5] supplemented with 10 mM potassium phosphate buffer (pH 6.8) and 0.1% yeast extract, in a glass fermentation vessel. Chemoheterotrophic (aerobic) cultures were vigorously aerated with 12 l per min of sterile air, and 250 rpm agitation. These cells were grown only to a density of approx. 2.4 $\times 10^8$ cells/ml to minimize the effects of oxygen limitation. The measurement of bacteriochlorophyll in such cultures confirmed that levels of this pigment were 300-fold lower than in cells grown photosynthetically.

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Medium for photoheterotrophic (anaerobic) growth of cells was purged of oxygen by vigorous bubbling with 5% CO_2 in N_2 for 0.5 h prior to inoculation. After inoculation the medium was sparged for 15 min before sealing the fermentor. Illumination was provided by a bank of nine 60 W Lumiline tungsten lamps encircling the vessel. The cells were harvested at a density of about 4×10^8 cells/ml by use of a Sharples centrifuge. The cell pellets were stored at −80°C immediately after centrifugation.

2.2. *Purification of RNA polymerase*

RNA polymerase was purified using a modified version of a published procedure [6]. The procedure was performed at 0–4°C and has been changed as described below. The fractions from the DNA-cellulose column containing activity were combined and condensed as before [6], diluted with buffer B (buffer A + 10% glycerol) to a conductivity of 5 mΩ (equivalent to buffer B containing 0.1 M NaCl), and applied to a 1 × 3 cm heparin-Sepharose column. The column was washed successively with 3 column volumes each of buffer B solution containing 0.1 M, 0.25 M and 0.35 M NaCl, and then the RNA polymerase activity was eluted with buffer B containing 0.6 M NaCl. For the additional purification 1 ml (150 μg) of RNA polymerase from the heparin-Sepharose eluate was applied to a column of DEAE-Sephadex. The enzyme was eluted with a 0.1–0.6 M NaCl gradient in buffer B.

2.3. *Preparation of DNA*

Salmon sperm DNA was obtained from Sigma (St. Louis). *R. capsulatus* and *Escherichia coli* chromosomal DNA, phage M13 and T_7 DNA, and pUC13 plasmid DNA were purified by CsCl gradient ultracentrifugation [7].

2.4. *RNA polymerase assays*

RNA polymerase assays for monitoring polymerase activity through its purification contained: 48 mM Tris-HCl, pH 8.2; 8 mM $MgCl_2$; 40 mM NaCl; 10% glycerol; 1.6 mM ATP, CTP, and GTP; 16 μM UTP; 28 nM [^{3}H]UTP (14 μCi/nmol); 5 μg salmon sperm DNA; and various amounts of RNA polymerase in a total volume of 500 μl. The assays were incubated at 34°C for 10 min and RNA was precipitated by addition of 1.0 ml of 10% trichloroacetic acid. One unit of activity was defined as 1 nmol of UTP in-

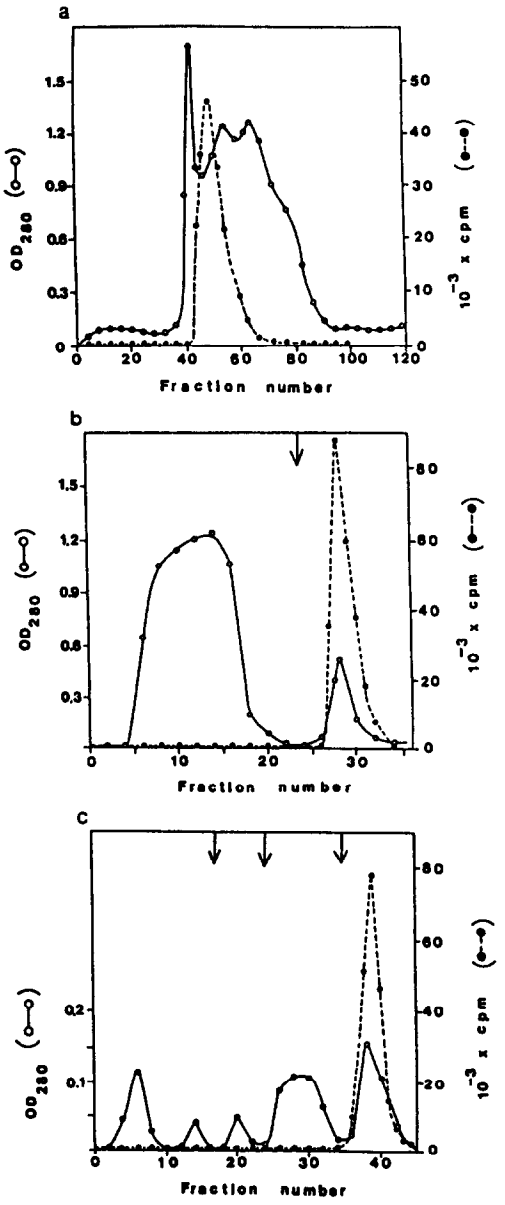

Fig.1. Chromatography of RNA polymerase from chemoheterotrophically grown cells: a, elution from Bio-Gel A1.5m; b, elution from DNA-cellulose; c, elution from heparin-Sepharose. The supernatant fluid applied to the columns was purified as described in section 2. Fractions were collected and assayed for RNA polymerase activity (●—●). The absorbance of the fractions at 280 nm was also measured (○—○).

corporated in 10 min. The amount of protein present was determined by measurement of Coomassie blue dye binding (Bio-Rad), with crystallized bovine serum albumin as standard. Specific activity was defined as units of activity per mg of protein.

Run-off transcription assays contained: 60 mM Tris-HCl, pH 8.2; 8 mM $MgCl_2$; 40 mM KCl; 13% glycerol; 1.6 mM CTP, UTP, and GTP; 16 μM ATP; 66 nM [^{32}P]ATP (756 μCi/nmol); 1 μg template DNA; and RNA polymerase in a 4:1 molar ratio of RNA polymerase:DNA, in a volume of 200 μl. The RNA polymerase and DNA were allowed to bind for 5 min at 34°C, then heparin and nucleotides were added. The assays were left at 34°C for 20 min. One tenth of the reaction was diluted into 1.0 ml of trichloroacetic acid for measurement of the amount of radioactivity incorporated into RNA, and the rest was extracted with an equal volume of phenol:chloroform (1:1), then with chloroform, and then, after the addition of 1/10 volume of 3 M sodium acetate, precipitated by addition of 0.5 ml of 95% ethanol.

2.5. *Polyacrylamide gel electrophoresis*

Slab gel electrophoresis of proteins was performed as described by Laemmli [8] with a 14–20% gradient of polyacrylamide [9]. Gel electrophoresis of RNA transcripts was done in 5% polyacrylamide-7 M urea gels, in 0.5 × TBE buffer [7]. After a 70% ethanol wash, the precipitated samples were dissolved in 20 μl of 80% formamide; 0.5 × TBE; 0.025% xylene cyanol; 0.025% bromophenol blue, and then heated for 5 min at 90°C before loading. Radiolabelled DNA from the single-stranded phage M13mp11, digested with *Hae*III, was used for molecular mass markers.

3. RESULTS

3.1. *Purification of RNA polymerase*

RNA polymerase was purified from both chemoheterotrophically and photoheterotrophically grown cells as described above. Fig.1 shows a typical elution of RNA polymerase activity from the Bio-Gel A 1.5m column, the DNA-cellulose column and the heparin-Sepharose column. Although salmon sperm DNA was routinely used as a template to monitor the polymerase activity throughout the purification, when *R. capsulatus* chromosomal DNA was used the results were comparable.

The results of a typical purification of RNA polymerase are summarized in table 1. The overall yield was usually between 10 and 15%, with an increase in specific activity of 100–200-fold. This yield is comparable to yields obtained by others in purification of bacterial RNA polymerases [10,11]. The final specific activity obtained was from 50 to 100 units/mg protein. Fractions containing the peak of activity from the heparin-Sepharose column were pooled and then separated into aliquots. The purified RNA polymerase was either stored at −20°C in the elution buffer (buffer B containing 0.6 M NaCl) supplemented with 40% glycerol, or

Table 1

Summary of RNA polymerase purification

Purification step	mg protein	Units of activity	Units/mg protein	% yield	Fold purification
Crude extract	2241	594	0.26	–	–
0–30% $(NH_4)_2SO_4$ supernatant	405	986	2.44	166	9.38
30–60% $(NH_4)_2SO_4$ pellet	294	642	2.18	108	8.38
Bio-Gel A1.5m eluate	67	495	7.40	83	28.5
DNA cellulose eluate	3.55	160	45.7	27	176
Heparin-Sepharose eluate	1.44	87	60.7	15	234

One unit of activity is defined as 1 nmol of UTP incorporated in 10 min at 34°C

at −80°C in the same buffer containing 10% glycerol. Specific activity declined during storage under both conditions over a period of 3–4 months to about 50% of the initial activity, and then stabilized.

A preliminary comparison of different templates for RNA polymerase activity was performed, and the results are shown in table 2. The greatest specific activity was obtained with phage M13RF as template whereas *R. capsulatus* DNA gave the lowest activity.

3.2. *Subunit composition of RNA polymerase*

The purified polymerase was subjected to SDS-PAGE to assess its subunit composition. Fig.2 shows the results of an experiment in which RNA polymerase preparations purified from aerobic and anaerobic cultures of *R. capsulatus* were compared to a preparation of *E. coli* RNA polymerase. The gel contained a number of bands representing proteins that were associated with RNA polymerase activity. Of the 10–11 visible bands, six were designated as RNA polymerase subunits because they consistently co-purified with RNA polymerase activity through two additional purification steps (see below). The designation and molecular mass (estimated by comparison with *E. coli* RNA polymerase subunits as standards) of the subunits are: β and β' subunits of about 150–160 kDa; an α subunit of about 45 kDa; and a σ subunit of 70 kDa. Two smaller proteins, tentatively designated as ω factors, were also consistently observed. The pattern of bands in the gel was identical for aerobic and anaerobic RNA polymerase preparations. However there was a much larger amount of one protein, which migrated to a position between the putative α and σ subunits, associated with the polymerase activity purified from cells grown aerobically.

Because of the large number of proteins present in the preparations analyzed in fig.2, aliquots of the polymerase were put through two additional

Table 2

Utilization of different templates by *R. capsulatus* RNA polymerase

Specific activity	DNA template
52	salmon sperm
35	T7
83	M13mp11
23	*E. coli*
9	*R. capsulatus*

Specific activity is in units/mg protein; assay conditions were those used for salmon sperm DNA (see section 2), 10 μg of each DNA template was used

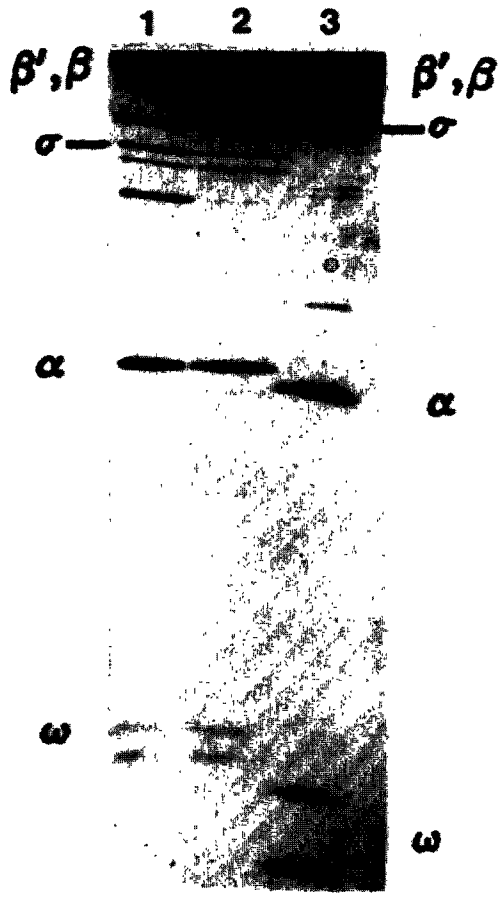

Fig.2. SDS-polyacrylamide gel electrophoresis of purified RNA polymerase from chemoheterotrophically and photoheterotrophically grown *R. capsulatus*. 10 μg protein from the heparin-Sepharose eluate, along with 10 μg *E. coli* RNA polymerase, were subjected to electrophoresis and stained as described in section 2. The subunit designations for *R. capsulatus* RNA polymerase are given on the left, and the subunits of *E. coli* RNA polymerase are labelled on the right. Lanes: 1, RNA polymerase from chemoheterotrophically grown cells; 2, RNA polymerase from photoheterotrophically grown cells; 3, *E. coli* RNA polymerase.

purifications to see if some of the proteins could be removed without loss of RNA polymerase activity. The methods used were ion-exchange chromatography over a DEAE-Sephadex column and centrifugation through a gradient of glycerol.

A single peak of activity was observed with chromatography over DEAE-Sephadex, and SDS-PAGE revealed about 7–8 proteins in the three fractions with greatest activity (fig.3). The specific activity of the peak fraction (lanes 3, fig.3) in the preparation from aerobic cells increased 1.5-fold, whereas the specific activity of the peak fraction of RNA polymerase from anaerobic cells remained the same. The results from the glycerol gradient were comparable (not shown). Only the gel bands that we have designated as being representative of *R. capsulatus* RNA polymerase subunits were visible in both preparations after both of the additional purification procedures, at levels consistent with the specific activities. On occasion extra bands were seen to co-purify with the RNA polymerase. These bands were not present in other purifications, and thus it is unlikely that they were components of the RNA polymerase that were essential for activity. There were differences in the number and amounts of contaminating proteins in different preparations, but the subunits that we have identified were always present.

3.3. *Gel electrophoresis of run-off transcription products*

The RNA polymerase preparations used for run-off transcriptions were obtained by elution from heparin-Sepharose with a step gradient of salt (see figs 1 and 2). Run-off transcription assays were performed as described in section 2. An example of

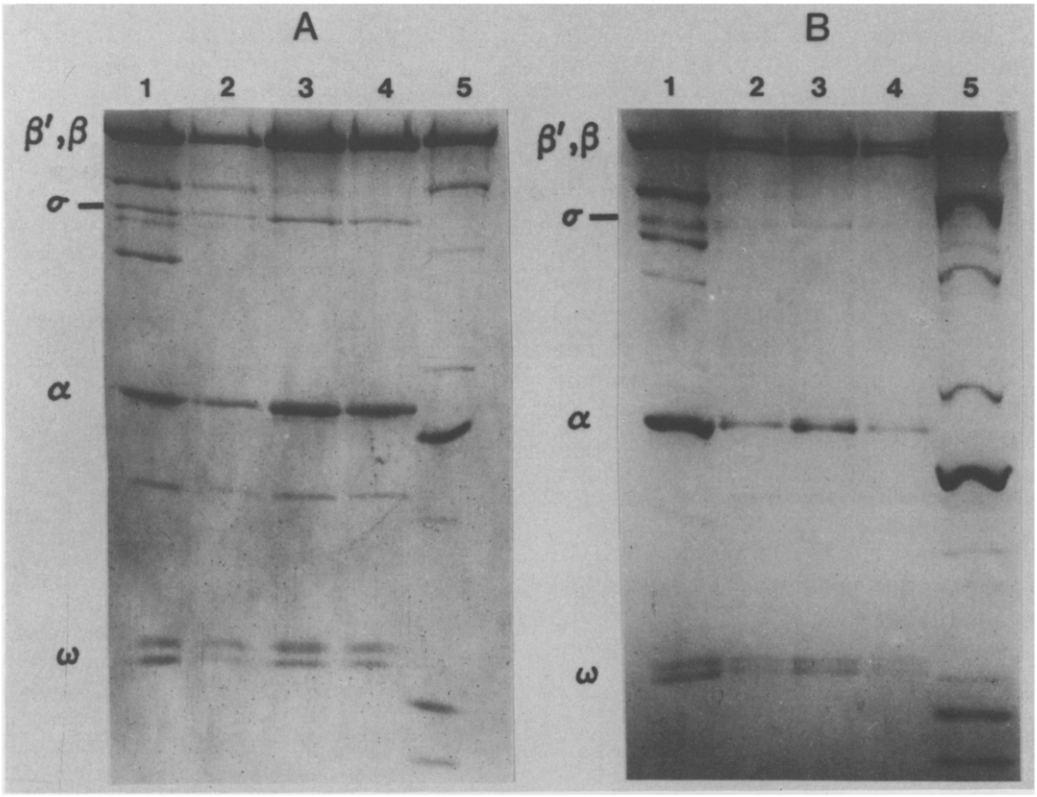

Fig.3. SDS-polyacrylamide gel electrophoresis of RNA polymerase after chromatography over a DEAE-Sephadex column. The subunit designations for *R. capsulatus* RNA polymerase are given on the left of each gel. (A) RNA polymerase from chemoheterotrophically grown cells; (B) RNA polymerase from photoheterotrophically grown cells. Lanes: 1, 10 μg RNA polymerase from the heparin-Sepharose eluate; 2–4, peak fractions from the DEAE-Sephadex column (equal volumes of each loaded with lane 3 containing 10 μg protein); 5, 10 μg *E. coli* RNA polymerase.

an autoradiogram of a polyacrylamide-urea gel of RNA transcripts produced by a preparation of RNA polymerase from anaerobically grown cells is shown in fig.4. The template consisted of 1 μg of the plasmid pUC13, linearized by digestion with *Bam*HI and *Hin*dIII, and various amounts of heparin were added to the standard reaction assay. It had been found previously that inclusion of heparin reduced a background that could obscure bands due to specific transcripts. About 3–4 distinct RNA species were visible over the entire range of heparin concentrations, up to 50 μg per ml. Especially notable was an approx. 104 nucleotide transcript that is probably RNA I [12]. The additional transcripts seen ranged from approx. 500 to about 2500 nucleotides in length. A longer exposure of the autoradiogram revealed no additional transcripts, but the resolution of the bands at the top of the gel was lost.

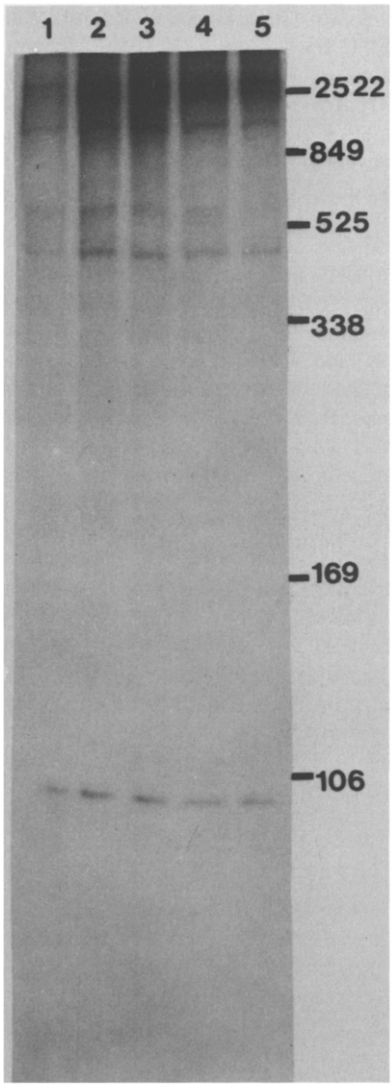

Fig.4. Polyacrylamide gel electrophoresis of run-off transcripts obtained with photoheterotrophic RNA polymerase. The template pUC13 was linearized with *Bam*HI and *Hin*dIII. Reaction conditions and gel electrophoresis were as described in section 2, with the following amounts of heparin added to the reactions: lanes: 1, 0.1 μg; 2, 0.3 μg; 3, 1.0 μg; 4, 3.0 μg; 5, 10.0 μg.

4. DISCUSSION

In this paper we describe the purification of RNA polymerase from *R. capsulatus* cells grown either chemoheterotrophically or photoheterotrophically. The RNA polymerase complex from *R. capsulatus* has a subunit composition similar to other eubacterial RNA polymerases that have been purified [10,13,14]. The putative β and β' subunits were very close in molecular mass to those of *E. coli*, and the putative α subunit seemed to be slightly larger than the 40 kDa *E. coli* α subunit. Although the polymerase activity that was obtained from the heparin-Sepharose column had several components that could have been the σ subunit(s), the two additional purifications performed removed most of these additional protein bands and prompted our provisional designation of σ. This component appears to be the same for RNA polymerase purified from chemoheterotrophically and photoheterotrophically grown *R. capsulatus*.

The RNA polymerase purified from the heparin-Sepharose column was the preparation used for in vitro transcription. Phage M13 differed from the other templates used in the testing of the polymerase activity in that it was supercoiled. The lower activity seen with *R. capsulatus* DNA, relative to the other linear templates, could be due to its higher GC content (approx. 67% as opposed to 41–50% for the other templates [15,16]). Radioactive RNA transcripts were made from linearized pUC13 as template and the transcripts were run on polyacrylamide gels. The transcripts showed that this preparation of RNA polymerase can initiate transcription efficiently at some *E. coli*

plasmid promoters.

The purification and preliminary characterization of *R. capsulatus* RNA polymerase has shown that the enzyme is similar to *E. coli* RNA polymerase in basic subunit structure. Both enzymes were capable of specific initiation and termination of transcription with linearized pUC13 DNA as template. However there must be some significant differences between *E. coli* and *R. capsulatus* RNA polymerase because some promoters show species specificity in vivo [17]. Further studies of the *R. capsulatus* RNA polymerase may now be performed with the use of fragments that contain photosynthesis gene promoters as template. Studies with this in vitro transcription system may provide information on how transcription of photosynthesis genes is controlled in response to environmental conditions.

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